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NOTIFICATION OF ELECTION

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Date of mailing (day/month/year) 01 September 2000 (01.09.00)	
International application No. PCT/CA99/01177	**Applicant's or agent's file reference** 1162/0051
International filing date (day/month/year) 10 December 1999 (10.12.99)	**Priority date** (day/month/year) 11 December 1998 (11.12.98)
Applicant SHIPMAN, Robert	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 July 2000 (04.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Manu Berrod
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

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Ernst & Young Tower
Suite 1800
222 Bay Street
P.O. Box 197, T.D. Centre
Toronto, Ontario M5K 1H6
CANADA

Date of mailing (day/month/year) 26 June 2000 (26.06.00)	
Applicant's or agent's file reference 1162/0051	**IMPORTANT NOTIFICATION**
International application No. PCT/CA99/01177	**International filing date** (day/month/year) 10 December 1999 (10.12.99)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address

DEETH WILLIAMS WALL
National Bank Building
Suite 400, 150 York Street
Toronto, Ontario M5H 3S5
Canada

State of Nationality:

State of Residence:

Telephone No.: 416 941 9440

Facsimile No.: 416 941 9443

Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☒ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address

DONAHUE ERNST & YOUNG
Ernst & Young Tower
Suite 1800
222 Bay Street
P.O. Box 197, T.D. Centre
Toronto, Ontario M5K 1H6
Canada

State of Nationality:

State of Residence:

Telephone No.: 416 943 2400

Facsimile No.: 416 943 2735

Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

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Telephone No.: (41-22) 338.83.38

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(71) Applicant *(for all designated States except US)*: VISIBLE GENETICS INC. [CA/CA]; 700 Bay Street, P.O. Box 333, Toronto, Ontario M5G 1Z6 (CA).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: SHIPMAN, Robert [CA/CA]; #2–6240 Montevideo Road, Mississauga, Ontario L5N 2N7 (CA).

(74) Agent: DEETH WILLIAMS WALL; National Bank Building, Suite 400, 150 York Street, Toronto, Ontario M5H 3S5 (CA).

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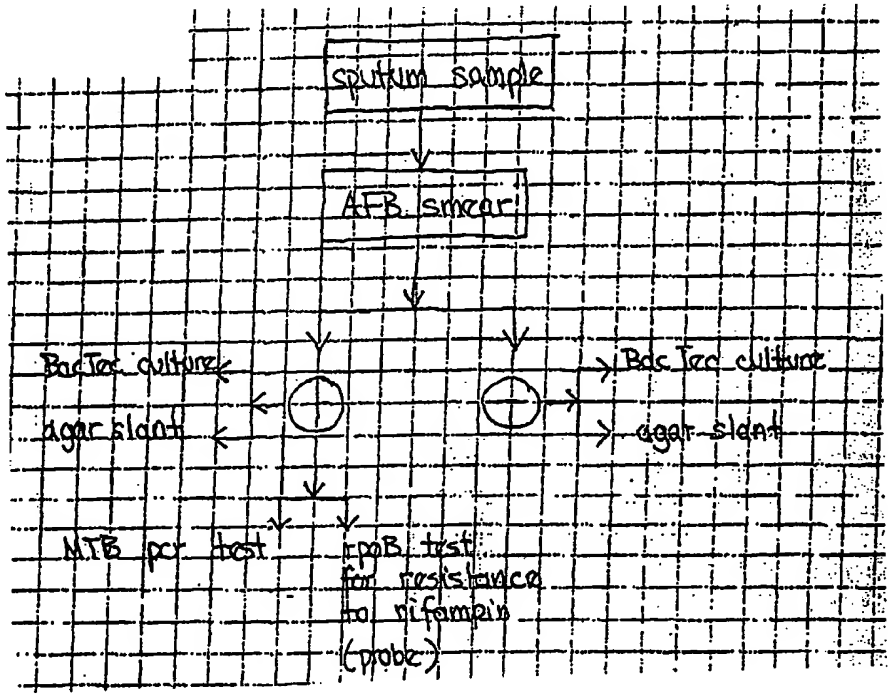
(54) Title: METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM* TUBERCULOSIS

(57) Abstract

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance–associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR–ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing M. tuberculosis, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic–resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic–resistance inducing mutations.



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INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract D71 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS:A MOLUCULAR STUDY" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ;LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ;HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ;BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM " ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. tional Application No
PCT/CA 99/01177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723650 A	03-07-1997	US 5834189 A	10-11-1998
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		EP 0870059 A	14-10-1998
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		CZ 9603612 A	16-07-1997
		EP 0771360 A	07-05-1997
		JP 10500857 T	27-01-1998

METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing it's uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of M. tuberculosis and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR–based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533[a]
2.	Isoniazid	katG gene	codon 275/315/328[b]
3.	Isoniazid	mabA gene	unknown[c]
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A[e]
6.	Pyrazinamide	pncA gene	codon 47/85[f]
7.	Ethambutol	embB gene	codon 306[g]
8.	Streptomycin	rpsL/s12 gene	codon 43/88[h]
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95[j]

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance–associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are know, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 2

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 4

SEQ. ID. NO. 5

```
2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc
2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc
2401 tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg
2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga
2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg
2581 tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg
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katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 9

SEQ ID NO. 10

```
661  gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca
721  gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc
781  ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc
841  gctgatcgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccgg ccgatctggt
901  cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta
961  tggcaccgga accggtaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac
1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac
```

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat
1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct
1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt
1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 11

PR-R amplification primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 12

PR-5s sequencing primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 13

PR-3s sequencing primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc
421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg
481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt
541 gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc
601 attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc
661 cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc
721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 16

mabA-R amplification primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 17

mabA-5s sequencing primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 18

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 19

SEQ ID NO. 20

```
1   agcgcgacat acctgctgcg caattcgtag ggcgtcaata cacccgcagc cagggcctcg
61  ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg
121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca
181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac
241 tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg
301 gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca
```

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO. 24

SEQ ID NO. 25

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1   cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag
61  gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac
121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg
181 agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg
241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc
301 cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc
361 gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca
```

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 29

SEQ ID NO. 30

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1   cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata
61  ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg
121 gcctatcagc ttgttggtgg ggtgacg
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embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 34

SEQ ID NO. 35

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7741 cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt
7801 gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat
7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt
7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt
7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct
```

pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 37

pncA-5s sequencing primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 38

pncA-3s sequencing primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 39

SEQ ID. NO. 40

```
1   atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg
61  gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac
241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggttt gcagctcctg a
```

gyrA (fluoroquinilone/ciprofloxacin resistance)

gyrA-F amplification primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 41

gyrA-R amplification primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 42

gyrA-5s sequencing primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 43

gyrA-3s sequencing primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 44

SEQ ID NO. 45

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2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa
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2521 gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat
2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg
2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc
2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg
2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg
2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga
2581 ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt
2641 tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a flourescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix		**1 PCR**	**10 PCRs**	**final conc. / PCR**
genomic DNA (~0.5fM)	(20ng/ul)	1.0ul		20ng
10X PCR buffer I		2.5ul	25.0ul	1X
2.5mM dNTP mix	(1:1:1:1)	2.5ul	25.0ul	250uM
DMSO		1.3ul	13.0ul	5%
Taq DNA polymerase	(1U)	0.2ul	2.0ul	1 unit
molecular grade water		16.5ul		165.0ul
MTB gene primers	(10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR		25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenace ™ buffer	2.5ul

DMSO	3.5ul
2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
1:10 diluted Thermosequenase	0.5 ul
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the *OPENGENE Automated DNA Sequencing System User Manual.* Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is it's limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect M. tuberculosis (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat M. tuberculosis infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada. Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

[a] **DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 38: 2380-2386.**

[b] **WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrob Agents Chemother 41: 1601-1603.**

[c] **S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and –susceptible Mycobacterium tuberculosis complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 41: 600-606.**

[d] **A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at the reference laboratory level. Antimicrob Agents Chemother 35: 719-723.**

[e] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[f] **C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.**

[g] **MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother 41: 2629-2633.**

[h] **A Scorpio et al. (1997). Characaterisation of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 41: 540-543.**

C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Disease 174: 1127-1130.

KA Nash et al. (1995). Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother 39: 2625-2630.

Table 1

gene (antiblotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcc65tct, Ser65Ser	wt	att133aat, Ile133Asn	wt	tcc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and

(c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs are selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

Figure 1.

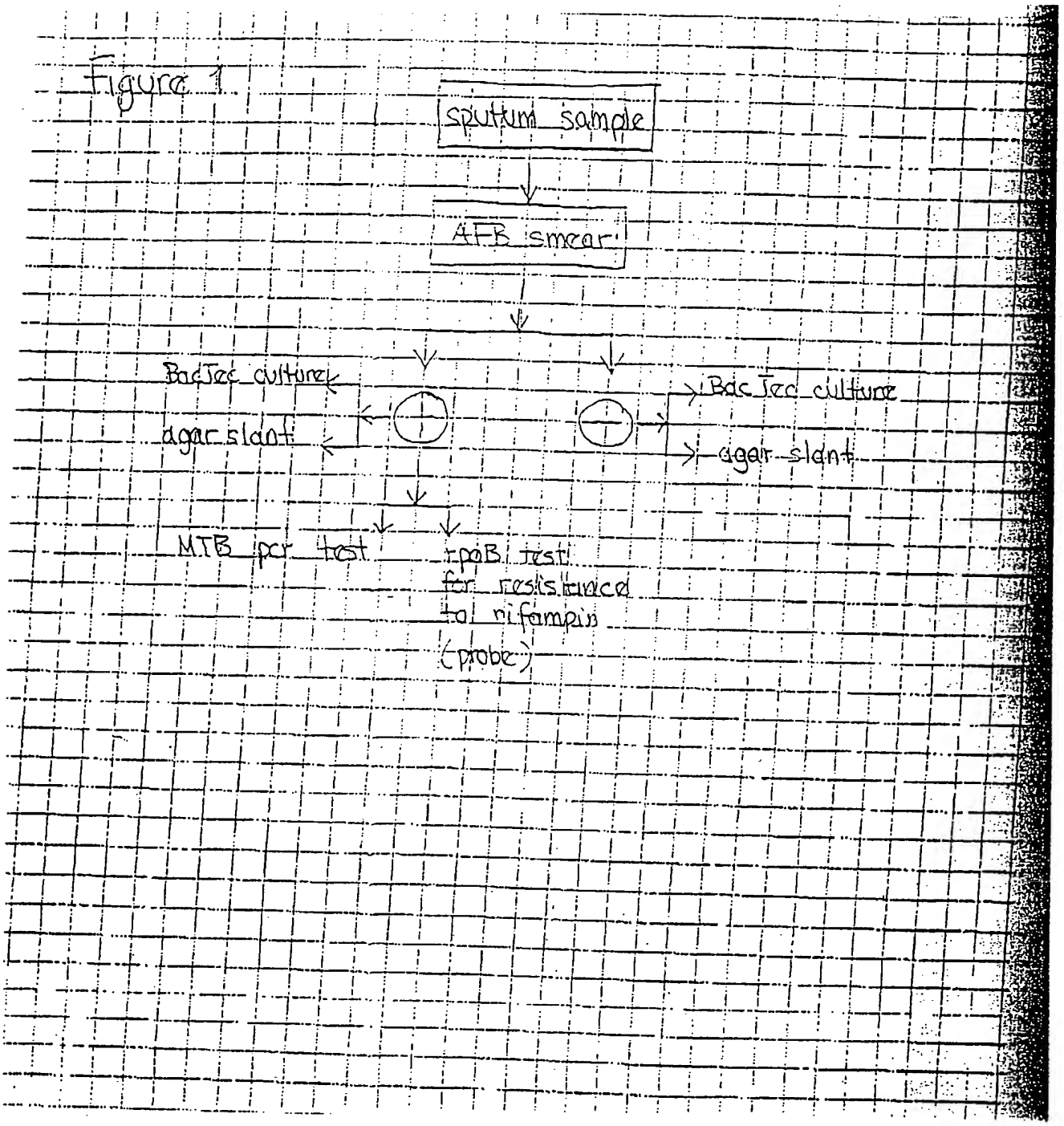
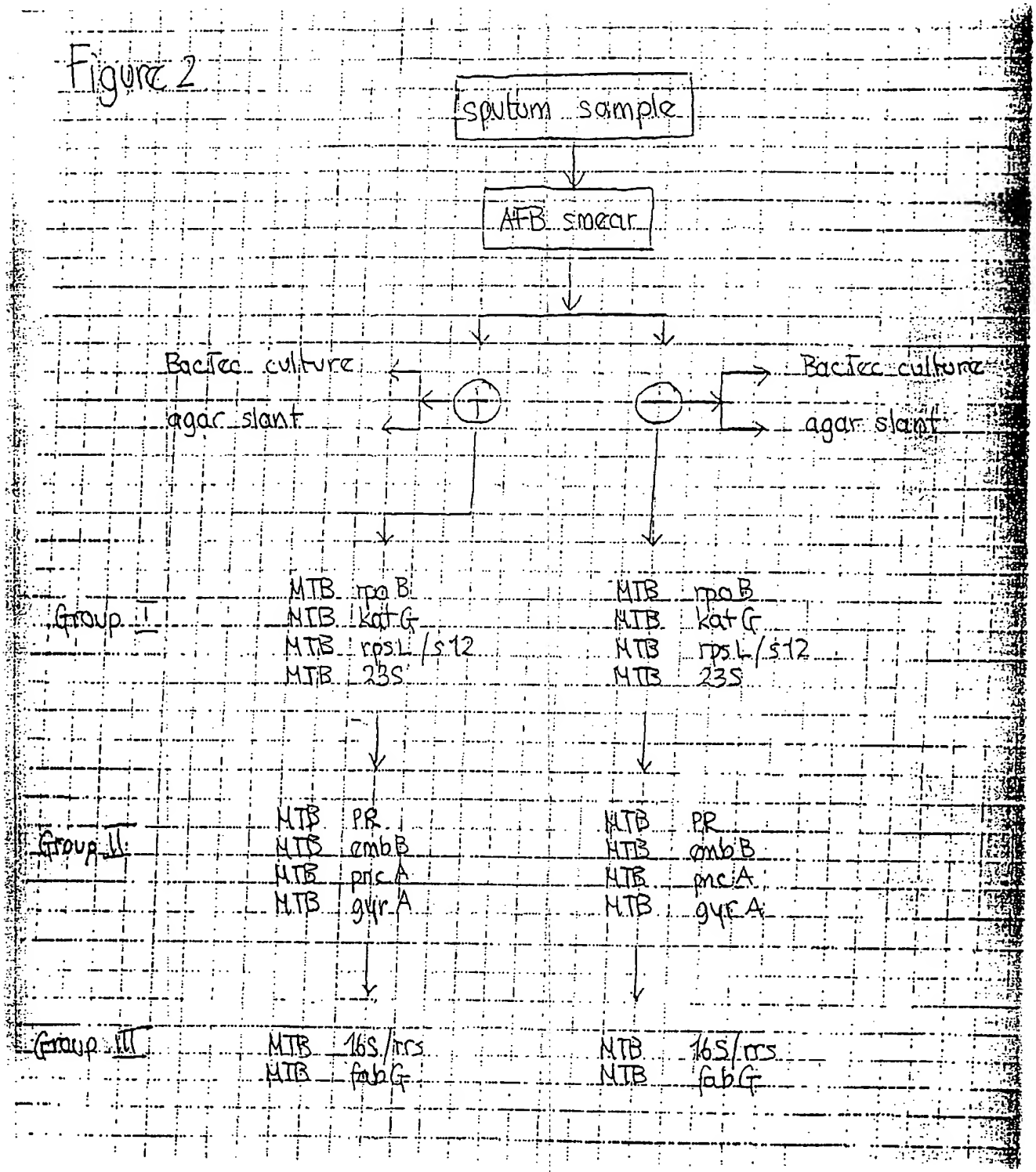

sputum sample
AFB smear
BacTec culture
agar slant
MTB pcr test
rpoB test for resistance to rifampin (probe)
BacTec culture
agar slant

Figure 2


sputum sample
AFB smear
BacTec culture
agar slant
BacTec culture
agar slant
Group I
MTB rpoB
MTB katG
MTB rpsL/s12
MTB 23S
MTB rpoB
MTB katG
MTB rpsL/s12
MTB 23S
Group II
MTB PR
MTB embB
MTB pncA
MTB gyrA
MTB PR
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MTB pncA
MTB gyrA
Group III
MTB 16S/rrs
MTB fabG
MTB 16S/rrs
MTB fabG

SEQUENCE LISTING

<110> Visible Genetics Inc.
Shipman, Robert

<120> Method and Kit for the Characterization of Antibiotic-Resistance Mutations in Mycobacterium tuberculosis

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<213> Mycobacterium tuberculosis

<220>
<223> rpoB-3s sequencing primer

<400> 4
tacggcgttt cgatgaaccc 20

<210> 5
<211> 480
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB (rifampin resistance)

<400> 5
aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc 60
aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca 120
cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg 180
cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc 240
tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg 300
agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga 360
tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg 420
tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg 480

<210> 6
<211> 20

<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-F amplification primer

<400> 6
atggggctga tctacgtgaa 20

<210> 7
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-R amplification primer

<400> 7
ggtgttccag ccagcgacgc 20

<210> 8
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-5s sequencing primer

<400> 8
atggggctga tctacgtgaa 20

<210> 9
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-3s sequencing primer

<400> 9
ggtgttccag ccagcgacgc 20

<210> 10
<211> 660
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG (isoniazid resistance)

<400> 10
gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca 60
gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc 120
ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc 180
gctgatcgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccgg ccgatctggt 240
cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta 300
tggcaccgga accggtaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac 360
cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac 420
gaagagcccc gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat 480
cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct 540
gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt 600
ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc 660

<210> 11
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-F amplification primer

<400> 11
accactgctt tgccgccacc 20

<210> 12
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-R amplification primer

<400> 12
ccgatgagag cggtgagctg 20

<210> 13
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-5s sequencing primer

<400> 13
accactgctt tgccgccacc 20

<210> 14
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-3s sequencing primer

<400> 14
ccgatgagag cggtgagctg 20

<210> 15
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> oxyR-ahpC intergenic region (PR)

<400> 15
atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc 60
tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg 120
cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt 180
gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc 240
attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc 300
cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc 360
cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt 420

<210> 16
<211> 20
<212> DNA

<213> Mycobacterium tuberculosis

<220>
<223> fabG-F amplification primer

<400> 16
cctcgctgcc cagaaaggga 20

<210> 17
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-R amplification primer

<400> 17
atcccccggt ttcctccggt 20

<210> 18
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-5s sequencing primer

<400> 18
cctcgctgcc cagaaaggga 20

<210> 19
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-3s sequencing primer

<400> 19
atcccccggt ttcctccggt 20

<210> 20
<211> 360
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG (isoniazid resistance)

<400> 20
agcgcgacat acctgctgcg caattcgtag ggcgtcaata cacccgcagc cagggcctcg 60
ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg 120
agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca 180
taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac 240
tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg 300
gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca 360

<210> 21
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-F amplification primer

<400> 21
cggtagatgc caaccatcca 20

<210> 22
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-R amplification primer

<400> 22
gcatcagccc ttctccttct 20

<210> 23
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-5s sequencing primer

<400> 23
cggtagatgc caaccatcca 20

<210> 24
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-3s sequencing primer

<400> 24
gcatcagccc ttctccttct 20

<210> 25
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpsL/s12 (streptomycin resistance)

<400> 25
cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag 60
gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac 120
accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg 180
agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg 240
atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc 300
cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc 360
gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca 420

<210> 26
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-F amplification primer

<400> 26
ggtgatctgc cctgcacttc g 21

<210> 27
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-R amplification primer

<400> 27
cgtcacccca ccaacaagct g 21

<210> 28
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-5s sequencing primer

<400> 28
ggtgatctgc cctgcacttc g 21

<210> 29
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-3s sequencing primer

<400> 29
cgtcacccca ccaacaagct g 21

<210> 30
<211> 147
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S/rrs (streptomycin resistance)

<400> 30
cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta taccggata 60
ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg 120
gcctatcagc ttgttggtgg ggtgacg 147

<210> 31
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-F amplification primer

<400> 31
cggcaagctg gcgcaccttc a 21

<210> 32
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-R amplification primer

<400> 32
agccagcaca ctagcccggc g 21

<210> 33
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-5s seuqencing primer

<400> 33
cggcaagctg gcgcaccttc a 21

<210> 34
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-3s sequencing primer

<400> 34
agccagcaca ctagcccggc g 21

<210> 35
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB (ethambutol resistance)

<400> 35
cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt 60
gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat 120
cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt 180
cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt 240
cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct 300

<210> 36
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 36
atgcgggcgt tgatcatcgt 20

<210> 37
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 37
tcaggagctg caaaccaact 20

<210> 38
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-5s sequencing primer

<400> 38
atgcgggcgt tgatcatcgt 20

<210> 39
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-3s sequencing primer

<400> 39
tcaggagctg caaaccaact 20

<210> 40
<211> 561
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA (pyrazinamide resistance)

<400> 40
atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg 60
gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac 120
taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc 180
acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc 300

ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg 360
cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc 420
cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca 480
gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc 540
gagttggttt gcagctcctg a 561

<210> 41
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-F amplification primer

<400> 41
cagctacatc gactatgcga 20

<210> 42
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-R amplification primer

<400> 42
gggcttcggt gtacctcatc 20

<210> 43
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-5s sequencing primer

<400> 43
cagctacatc gactatgcga 20

<210> 44
<211> 20

<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-3s sequencing primer

<400> 44
gggcttcggt gtacctcatc 20

<210> 45
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA (fluoroquinilone/ciprofloxacin resistance)

<400> 45
cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc 60
gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca 120
tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa 180
gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat 240
ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg 300
ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc 360
ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt 420

<210> 46
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-F amplification primer

<400> 46
cgaaattcct tgtcgggtaa 20

<210> 47
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-R amplification primer

<400> 47
gtatttcaac aacgactcca 20

<210> 48
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-5s sequencing primer

<400> 48
cgaaattcct tgtcgggtaa 20

<210> 49
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-3s sequencing primer

<400> 49
gtatttcaac aacgactcca 20

<210> 50
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S (macrolide/azithromycin resistance)

<400> 50
gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg 60
taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg 120
cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga 180
ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg 300

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(71) Applicant *(for all designated States except US)*: VISIBLE GENETICS INC. [CA/CA]; 700 Bay Street, P.O. Box 333, Toronto, Ontario M5G 1Z6 (CA).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: SHIPMAN, Robert [CA/CA]; #2-6240 Montevideo Road, Mississauga, Ontario L5N 2N7 (CA).

(74) Agent: DONAHUE ERNST & YOUNG; Ernst & Young Tower, Suite 1800, 222 Bay Street, P.O. Box 197, T.D. Centre, Toronto, Ontario M5K 1H6 (CA).

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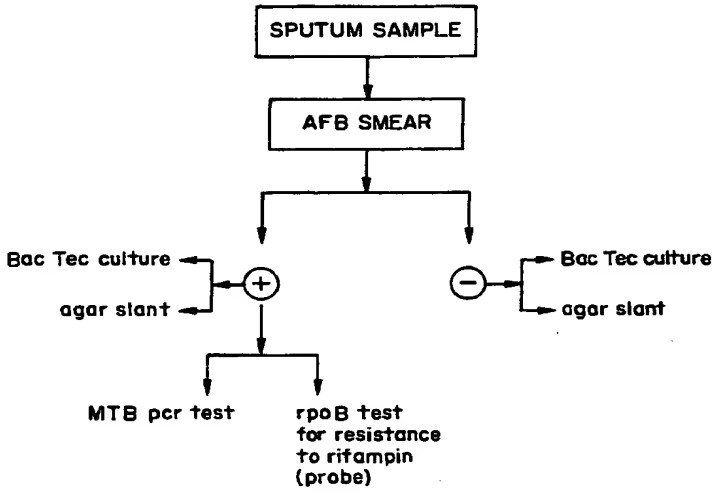
(54) Title: METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*



(57) Abstract: Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing M. tuberculosis, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

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METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN MYCOBACTERIUM TUBERCULOSIS

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing it's uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for M. tuberculosis, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of M. tuberculosis and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR-based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of M. tuberculosis has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533a
2.	Isoniazid	katG gene	codon 275/315/328b
3.	Isoniazid	mabA gene	unknown c
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A e
6.	Pyrazinamide	pncA gene	codon 47/85 f
7.	Ethambutol	embB gene	codon 306 g
8.	Streptomycin	rpsL/s12 gene	codon 43/88 h
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95 j

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical M. tuberculosis sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in M. tuberculosis.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), mabA (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance-associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture M. tuberculosis and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for M. tuberculosis; and

Fig. 2 shows a hierarchical assay scheme for evaluating M. tuberculosis type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are know, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 2

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3' SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3' SEQ ID NO. 4

SEQ. ID. NO. 5

2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc

2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca

2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg

2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc

2401 tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg

2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga

2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg

2581 tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3' SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3' SEQ ID NO. 9

SEQ ID NO. 10

661 gctcggcgat gagcgttaca gcggtaagcg ggatctggag aacccgctgg ccgcggtgca

721 gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc

781 ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc

841 gctgatcgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccgg ccgatctggt

901 cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta

961 tggcaccgga accggtaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac

1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat

1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct

1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt

1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 11

PR-R amplification primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 12

PR-5s sequencing primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3' SEQ ID NO. 13

PR-3s sequencing primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3' SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc

421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg

481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt

541 gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc

601 attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc

661 cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc

721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 16

mabA-R amplification primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 17

mabA-5s sequencing primer. 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3' SEQ ID NO. 18

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3' SEQ ID NO. 19

SEQ ID NO. 20

1 agcgcgacat acctgctgcg caattcgtag ggcgtcaata cacccgcagc cagggcctcg

61 ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg

121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca

181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac

241 tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg

301 gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3' SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3' SEQ ID NO. 24

SEQ ID NO. 25

1 cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag

61 gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac

121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg

181 agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg

241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc

301 cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc

361 gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3' SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3' SEQ ID NO. 29

SEQ ID NO. 30

1 cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta taccggata

61 ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg

121 gcctatcagc ttgttggtgg ggtgacg

embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3' SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3 SEQ ID NO. 34

SEQ ID NO. 35

7741 cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt

7801 gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat

7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt

7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt

7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct

pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542
5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 37
pncA-5s sequencing primer, 20-mer, bp1-20
5' ATG CGG GCG TTG ATC ATC GT 3' SEQ ID NO. 38
pncA-3s sequencing primer, 20-mer, bp561-542
5' TCA GGA GCT GCA AAC CAA CT 3' SEQ ID NO. 39

SEQ ID. NO. 40

1 atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg
61 gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac
241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggttt gcagctcctg a

gyrA (fluoroquinilone/ciprofloxacin resistance)
gyrA-F amplification primer, 20-mer, bp2383-2402
5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 41
gyrA-R amplification primer, 20-mer, bp2702-2683
5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 42
gyrA-5s sequencing primer, 20-mer, bp2383-2402
5' CAG CTA CAT CGA CTA TGC GA 3' SEQ ID NO. 43
gyrA-3s sequencing primer, 20-mer, bp2702-2683
5' GGG CTT CGG TGT ACC TCA TC 3' SEQ ID NO. 44

SEQ ID NO. 45

2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa

2521 gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat

2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg

2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc

2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3' SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3' SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg

2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg

2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga

2581 ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt

2641 tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a flourescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix	**1 PCR**	**10 PCRs**	**final conc. / PCR**
genomic DNA (20ng/ul)	1.0ul		20ng (~0.5fM)
10X PCR buffer I	2.5ul	25.0ul	1X
2.5mM dNTP mix (1:1:1:1)	2.5ul	25.0ul	250uM
DMSO	1.3ul	13.0ul	5%
Taq DNA polymerase (1U)	0.2ul	2.0ul	1 unit
molecular grade water	16.5ul	165.0ul	
MTB gene primers (10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR	25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template 2.0ul

10X VGI Sequenace (tm) buffer 2.5ul

DMSO		3.5ul
2.5uM dye-sequencing primer	2.0ul	
PCR grade water		9.0ul
1:10 diluted Thermosequenase	0.5 ul	
total volume		22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the ***OPENGENE Automated DNA Sequencing System User Manual***. Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™ . This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is it's limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect M. tuberculosis (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat *M. tuberculosis* infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of M. tuberculosis (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of M. tuberculosis from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada. Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

a DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 38: 2380-2386.

b WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of Mycobacterium tuberculosis complex from Africa. Antimicrob Agents Chemother 41: 1601-1603.

c S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and -susceptible Mycobacterium tuberculosis complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 41: 600-606.

d A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at the reference laboratory level. Antimicrob Agents Chemother 35: 719-723.

e C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.

f C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of Mycobacterium tuberculosis in Japan. J Appl Microbiol 83: 634-640.

g MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother 41: 2629-2633.

h A Scorpio et al. (1997). Characaterisation of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 41: 540-543.

i C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Disease 174: 1127-1130.

j KA Nash et al. (1995). Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother 39: 2625-2630.

Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcc65tct, Ser65Ser	wt	att133aat, Ile133Asn	wt	tcc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and

(c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of M. tuberculosis, characterized in that the amplification and sequencing primer pairs are selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

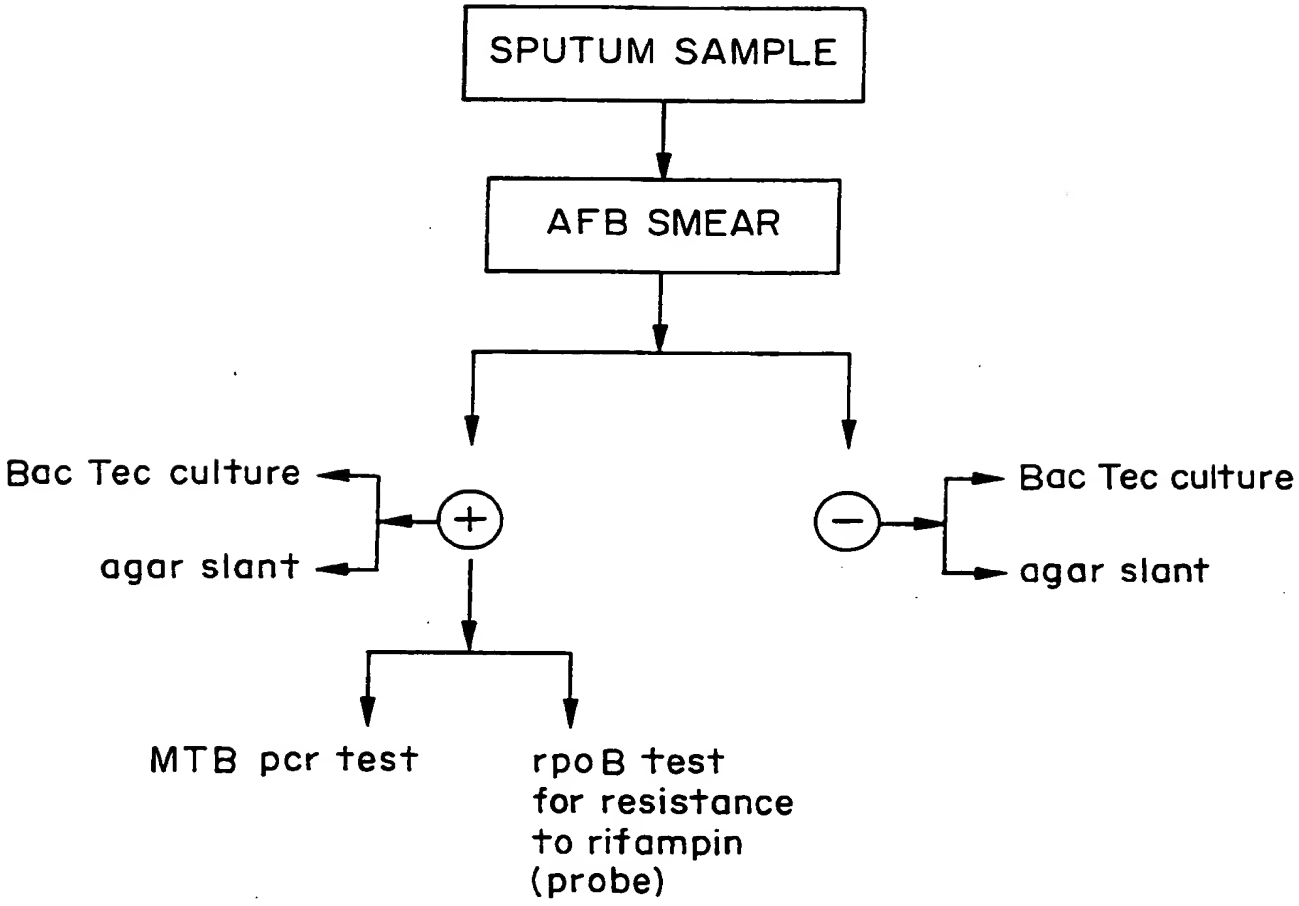
SPUTUM SAMPLE
AFB SMEAR
Bac Tec culture
agar slant
+
−
Bac Tec culture
agar slant
MTB pcr test
rpoB test
for resistance
to rifampin
(probe)

FIG. 1

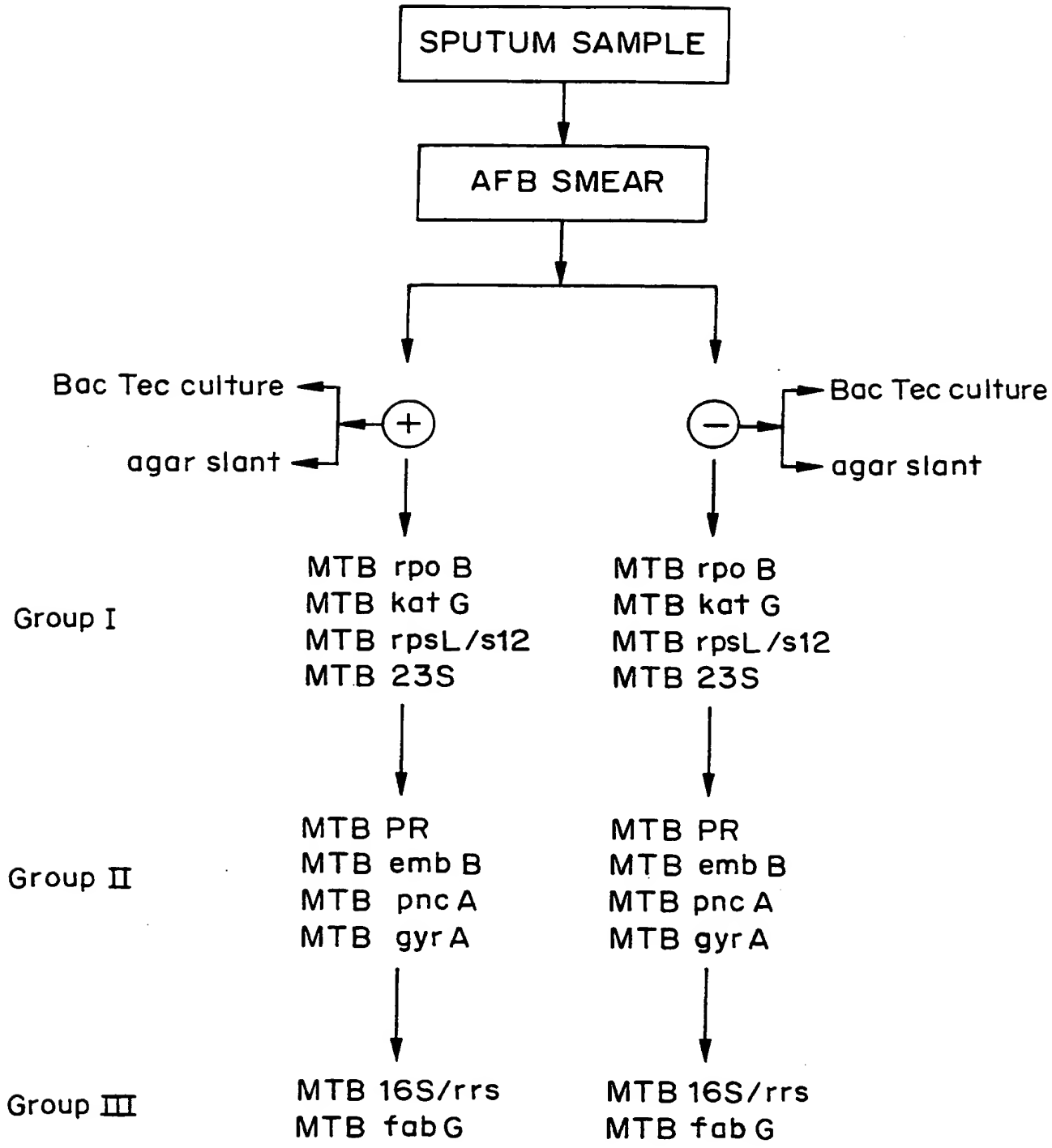
SPUTUM SAMPLE
AFB SMEAR
Bac Tec culture
agar slant
+
−
Bac Tec culture
agar slant
Group I
MTB rpo B
MTB kat G
MTB rpsL/s12
MTB 23S
MTB rpo B
MTB kat G
MTB rpsL/s12
MTB 23S
Group II
MTB PR
MTB emb B
MTB pnc A
MTB gyr A
MTB PR
MTB emb B
MTB pnc A
MTB gyr A
Group III
MTB 16S/rrs
MTB fab G
MTB 16S/rrs
MTB fab G

FIG. 2

SEQUENCE LISTING

<110> Visible Genetics Inc.
Shipman, Robert

<120> Method and Kit for the Characterization of Antibiotic-Resistance Mutations in Mycobacterium tuberculosis

<130> VGEN.P-055-WO

<140>
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<151> 1998-12-11

<160> 50

<170> PatentIn Ver. 2.1

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<220>
<223> rpoB-F amplification primer

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<210> 2
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<220>
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<400> 2
tacggcgttt cgatgaaccc 20

<210> 3
<211> 20
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<213> Mycobacterium tuberculosis

<220>
<223> rpoB-5s sequencing primer

<400> 3
tacggtcggc gagctgatcc 20

<210> 4
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB-3s sequencing primer

<400> 4
tacggcgttt cgatgaaccc 20

<210> 5
<211> 480
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB (rifampin resistance)

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aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca 120
cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg 180
cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc 240
tgtcggggtt gacccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg 300
agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga 360
tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcggtg tacgcgcggg 420
tcaacccgtt cgggttcatc gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg 480

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<220>
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atggggctga tctacgtgaa 20

<210> 7
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-R amplification primer

<400> 7
ggtgttccag ccagcgacgc 20

<210> 8
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<220>
<223> katG-5s sequencing primer

<400> 8
atggggctga tctacgtgaa 20

<210> 9
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-3s sequencing primer

<400> 9
ggtgttccag ccagcgacgc 20

<210> 10
<211> 660
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG (isoniazid resistance)

<400> 10
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gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc 120
ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc 180
gctgatcgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccgg ccgatctggt 240
cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta 300
tggcaccgga accggtaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac 360
cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac 420
gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat 480
cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct 540
gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt 600
ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc 660

<210> 11
<211> 20
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<213> Mycobacterium tuberculosis

<220>
<223> PR-F amplification primer

<400> 11
accactgctt tgccgccacc 20

<210> 12
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-R amplification primer

<400> 12
ccgatgagag cggtgagctg 20

<210> 13
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-5s sequencing primer

<400> 13
accactgctt tgccgccacc 20

<210> 14
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-3s sequencing primer

<400> 14
ccgatgagag cggtgagctg 20

<210> 15
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> oxyR-ahpC intergenic region (PR)

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tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg 120
cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt 180
gatatatcac ctttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc 240
attgtccgct ttgatgatga ggagagtcat gccactgcta accattggcg atcaattccc 300
cgcctaccag ctcaccgctc tcatcggcgg tgacctgtcc aaggtcgacg ccaagcagcc 360
cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc gggtggtgtt 420

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<220>
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cctcgctgcc cagaaaggga 20

<210> 17
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-R amplification primer

<400> 17
atcccccggt ttcctccggt 20

<210> 18
<211> 20
<212> DNA
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<220>
<223> fabG-5s sequencing primer

<400> 18
cctcgctgcc cagaaaggga 20

<210> 19
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-3s sequencing primer

<400> 19
atcccccggt ttcctccggt 20

<210> 20
<211> 360
<212> DNA
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<220>
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ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg 120
agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgcag ccacgttacg ctcgtggaca 180
taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac 240
tgaaggggcc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg 300
gatcgggctg gcgatcgcac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca 360

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<211> 20
<212> DNA
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<220>
<223> s12-F amplification primer

<400> 21
cggtagatgc caaccatcca 20

<210> 22
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-R amplification primer

<400> 22
gcatcagccc ttctccttct 20

<210> 23
<211> 20
<212> DNA
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<220>
<223> s12-5s sequencing primer

<400> 23
cggtagatgc caaccatcca 20

<210> 24
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-3s sequencing primer

<400> 24
gcatcagccc ttctccttct 20

<210> 25
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpsL/s12 (streptomycin resistance)

<400> 25
cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag 60
gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac 120
accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcgt gaagttgacg 180
agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg 240
atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc 300
cgcggttcgc tggatacgca gggtgtcaag aaccgcaaac aggcacgcag ccgttacggc 360
gctaagaagg agaagggctg atgccacgca aggggcccgc gcccaagcgt ccgttggtca 420

<210> 26
<211> 21
<212> DNA
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<220>
<223> 16S-F amplification primer

<400> 26
ggtgatctgc cctgcacttc g 21

<210> 27
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-R amplification primer

<400> 27
cgtcacccca ccaacaagct g 21

<210> 28
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-5s sequencing primer

<400> 28
ggtgatctgc cctgcacttc g 21

<210> 29
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-3s sequencing primer

<400> 29
cgtcacccca ccaacaagct g 21

<210> 30
<211> 147
<212> DNA
<213> Mycobacterium tuberculosis

<220>
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<400> 30
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ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg 120
gcctatcagc ttgttggtgg ggtgacg 147

<210> 31
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<220>
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<400> 31
cggcaagctg gcgcaccttc a 21

<210> 32
<211> 21
<212> DNA

<213> Mycobacterium tuberculosis

<220>
<223> embB-R amplification primer

<400> 32
agccagcaca ctagcccggc g 21

<210> 33
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
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<400> 33
cggcaagctg gcgcaccttc a 21

<210> 34
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<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-3s sequencing primer

<400> 34
agccagcaca ctagcccggc g 21

<210> 35
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB (ethambutol resistance)

<400> 35
cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt 60
gatattcggc ttcctgctct ggcatgtcat cggcgcgaat tcgtcggacg acggctacat 120
cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctggtt 180
cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt 240
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<220>
<223> pncA-F amplification primer

<400> 36
atgcgggcgt tgatcatcgt 20

<210> 37
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 37
tcaggagctg caaaccaact 20

<210> 38
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-5s sequencing primer

<400> 38
atgcgggcgt tgatcatcgt 20

<210> 39
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-3s sequencing primer

<400> 39
tcaggagctg caaaccaact 20

<210> 40
<211> 561
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA (pyrazinamide resistance)

<400> 40
atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctcgctggcg 60
gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac 120
taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc 180
acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc 300
ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg 360
cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc 420
cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggt ggacctgaca 480
gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc 540
gagttggttt gcagctcctg a 561

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<220>
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<400> 41
cagctacatc gactatgcga 20

<210> 42
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<220>
<223> gyrA-R amplification primer

<400> 42
gggcttcggt gtacctcatc 20

<210> 43
<211> 20

<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-5s sequencing primer

<400> 43
cagctacatc gactatgcga 20

<210> 44
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA-3s sequencing primer

<400> 44
gggcttcggt gtacctcatc 20

<210> 45
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> gyrA (fluoroquinilone/ciprofloxacin resistance)

<400> 45
cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc 60
gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca 120
tcgccgggtg ctctatgcaa tgttcgattc cggcttccgc ccggaccgca gccacgccaa 180
gtcggcccgg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat 240
ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tggtggacgg 300
ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc 360
ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt 420

<210> 46
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<213> Mycobacterium tuberculosis

<220>
<223> 23S-F amplification primer

<400> 46
cgaaattcct tgtcgggtaa 20

<210> 47
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-R amplification primer

<400> 47
gtatttcaac aacgactcca 20

<210> 48
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-5s sequencing primer

<400> 48
cgaaattcct tgtcgggtaa 20

<210> 49
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S-3s sequencing primer

<400> 49
gtatttcaac aacgactcca 20

<210> 50
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 23S (macrolide/azithromycin resistance)

<400> 50
gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg 60
taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg 120
cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccggga 180
ccttcactac aacttggtat tggtgttcgg tacggtttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg 300

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations; and

(c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs are selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

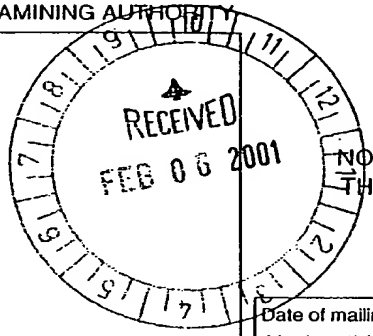
(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

DEETH WILLIAMS WALL
National Bank Building
150 York Street, Suite 400
Toronto, Ontario M5H 3S5
CANADA



PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	29.01.2001
Applicant's or agent's file reference: 2393-001205	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01177	10/12/1999	11/12/1998

Applicant
VISIBLE GENETICS INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Pedersen, C Tel.+49 89 2399-8663 8161

Form PCT/IPEA/416 (July 1992)

09/786105
5620

5000

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2393-001205		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01177	10/12/1999	11/12/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant VISIBLE GENETICS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/07/2000	29.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Knudsen, H Telephone No. +49 89 2399 8696

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/01177

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1,3-18	as originally filed	
2	with telefax of	10/01/2001

Claims, No.:

1-14	with telefax of	10/01/2001

Drawings, sheets:

1/2,2/2	as originally filed

Sequence listing part of the description, pages:

1-15, filed with the letter of 05.06.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☒ contained in the international application in written form.
   - ☒ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-14
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-14
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

ITEM I:

The sequence listing pages 1-15, filed with the letter of 05.06.2000 do not form part of the application (Rule 13ter.1(f) PCT).

ITEM II:

The priority appears to be validly claimed for the whole claimed subject-matter and the P-documents mentioned in the International Search Report therefore do not appear to be relevant. The applicant explains that the designation "fabG" used in the priority document and the designation "mabA" used in the application are equivalents and that the sole cause for making the amendment was the wish to use updated language.

ITEM V:

THE CLOSEST PRIOR ART:

5.1 The closest prior art is disclosed in "Abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy, vol. 34, page 163, (1994)" (D1). D1 discloses a method for identification of mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis by application of automated DNA sequence analysis. In the method a smear test is first carried out followed by an identification of a species specific hsp65 polymorphism. Subsequently, mutations were found in specific regions of seven genes. The genes investigated in D1 are rpoB, katG, inhA, orf1, rpsL, gyrA and 16SrRNA.

NOVELTY:

5.2 The method of claim 1 differs from the method of D1 in that the rpoB primers are selected so that an amplification of a sequence from the rpoB gene is indicative of the presence of Mycobacterium tuberculosis and in that the 23S gene is among the genes sequenced at the same time as the species-specific sequence and in that the sequences of the PR, embB, pncA and gyrA sequences are determined if the species specific test is positive. Thus, claims 1-13 are considered novel.

5.3 The only primer sequence of the present application, which is known from the prior art, is SEQ ID NO.1 which is disclosed in claim 22 of WO 95/33851 (D2). However, none of the combination of amplification and sequencing pairs are disclosed in the cited prior art documents. Claim 14 is therefore considered novel.

INVENTIVE STEP:

5.4 All of the genes mentioned in claims 1-3 of the present application are well-known as being involved in resistance development in Mycobacterium tuberculosis.

To the skilled person, who wishes to detect and characterize the resistance profile of a Mycobacterium tuberculosis sample isolated from an infected patient, it would therefore, prima facie, be obvious to test whether one or more of these genes carries a mutation. D1 informs the skilled person that mutations may be identified by sequencing of a number of genes. WO 97/23650 (D3) provides detailed information on how to sequence for polymorphisms.

WO 95/33074 (D2) discloses that PCR of a region of the rpoB gene may be used for both M.tuberculosis detection and drug susceptibility testing. The feature of claim 1 that the generation of rpoB sequences is indicative of the presence of M.tuberculosis therefore does not contribute to the inventiveness of claim 1. However, none of the cited documents suggest that the sequence of the 23S gene is useful in species determination of other Mycobacteria strains, in case the test for M.tuberculosis is negative. The method of claim 1 therefore solves the problem of providing in a single sequencing procedure the possibility of detecting and drug susceptibility testing M.tuberculosis and alternatively identifying another mycobacterium strains. In the absence of any suggestion in the prior art to include the 23S gene in the genes to be analysed firstly in a hierachical method, claim 1 is considered inventive.

Claims 2-13 which depend on claim 1 are considered inventive as well.

5.5 The use of primers for amplification of genes involved in antibiotic resistance is disclosed in D1 and is not considered inventive. The special combinations of amplification primers and sequencing primers (a) - (j) mentioned in claim 14 are not suggested in the prior art, but the sets of primers, per se, do not appear to

overcome a problem of the prior art documents which disclose methods for determining the sequence of several M.tuberculosis genes. The applicant argues that some primers perform better than others and considers the primers mentioned in claim 14 to perform very well. However, nothing in the present application suggests that any of the sets of primers solve a technical problem in an unexpected way. Nevertheless, the inclusion of 23S in the genes to be sequenced is not suggested in D1 and given the advantage obtained by its inclusion, present claim 14 is considered inventive.

INDUSTRIAL APPLICABILITY:

5.6 Present claims 1-14 are considered industrially applicable.

ITEM VII:

7.1 Contrary to the requirements of Rule 5(a)(ii) PCT, the discussion of the closest prior art document D1 does not reflect all the relevant background art disclosed therein.

7.2 It is not possible to incorporate the teaching of a prior art document into the present application's disclosure by the expression "herein incorporated by reference" or equivalents thereof (see p.4) (cf PCT Guidelines, C-II, 4.17).

ITEM VIII:

8.1 It is not clear from claim 3 under which conditions the third sequencing procedure is carried out, if the mere presence of M.tuberculosis is sufficient to give rise to the third sequencing procedure, then the prerequisites for carrying out the third and second sequencing procedures are the same and the hierarchical principle of the invention as explained in the application is not observed.

8.2 The object of the invention would only appear to be met if the kit contains primers which amplify the rpoB gene in a way so that the amplification of the rpoB gene is indicative of the presence of M.tuberculosis. At present this is not a requirement of claim 14.

8.3 It is not clear from the application whether the sequencing of a special region of

the 23S gene is needed in order to identify other strains than M.tuberculosis.

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533[a]
2.	Isoniazid	katG gene	codon 275/315/328[b]
3.	Isoniazid	mabA gene	unknown[c]
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A[e]
6.	Pyrazinamide	pncA gene	codon 47/85[f]
7.	Ethambutol	embB gene	codon 306[g]
8.	Streptomycin	rpsL/s12 gene	codon 43/88[h]
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95[j]

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in it's present form.

Abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy", vol. 34, page 163 (1994) describes the application of automated DNA sequence analysis of *hsp65* to speciation of isolates previously-identified as being *M. tuberculosis*.

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:

(a) obtaining a sputum sample suspected of containing *M. tuberculosis*,

(b) performing a first sequencing procedure, with or without prior amplification, on the sample, said sequencing procedure generating sequencing fragments for evaluation of the rpoB, katG, rpsL/s12 and 23S genes for the presence of antibiotic-resistance inducing mutations when *M. tuberculosis* is present in the sample, wherein primers for the sequencing of the rpoB gene are selected such that the generation of sequencing products for this gene is indicative of the presence of *M. tuberculosis* in the sample; and

(c) if *M. tuberculosis* is detected as a result of generation of sequencing products for the rpoB gene in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate at least one additional *M. tuberculosis* gene for the presence of antibiotic-resistance inducing mutations.

2. The method of claim 1, wherein the second sequencing procedure evaluates PR, embB pncA and gyrA genes for the presence of antibiotic-resistance mutations.

3. The method of claim 1, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b), separate from the first and second sequencing procedures, to evaluate 16S/rrs and mabA genes for the presence of antibiotic-resistance mutations.

4. The method of any of claims 1 to 3, wherein the first sequencing procedure for rpoB is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.

5. The method of any of claims 1 to 4, wherein the first sequencing procedure for katG is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.

6. The method of any of claims 1 to 5, wherein the first sequencing procedure for rpsL/s12 is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising pairs of amplification primers and matched pairs of sequencing primers for amplification and sequencing the at least the rpoB, katG, rpsL/s12 and 23S genes of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs include at least one combination of primer pairs selected from among:

(a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;

(b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;

(c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;

(d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;

(e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;

(f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;

(g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;

(h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;

(i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and

(j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
1162/0051		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 99/ 01177	10/12/1999	11/12/1998
Applicant		
VISIBLE GENETICS INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract D71 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document ---	1-3

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS:A MOLUCULAR STUDY" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ;LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ;HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ;BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM " ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13 -----	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 99/01177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723650 A	03-07-1997	US 5834189 A	10-11-1998
		AU 1426297 A	17-07-1997
		CA 2239896 A	03-07-1997
		EP 0870059 A	14-10-1998
		WO 9741257 A	06-11-1997
		US 5888736 A	30-03-1999
		US 5981186 A	09-11-1999
		US 6007983 A	28-12-1999
WO 9533074 A	07-12-1995	US 5643723 A	01-07-1997
		AU 2605795 A	21-12-1995
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PATENT COOPERATION TRE TY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2393-001205		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01177	10/12/1999	11/12/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant VISIBLE GENETICS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/07/2000	29.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Knudsen, H Telephone No. +49 89 2399 8696

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/01177

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1,3-18	as originally filed	
2	with telefax of	10/01/2001

Claims, No.:

1-14	with telefax of	10/01/2001

Drawings, sheets:

1/2,2/2	as originally filed

Sequence listing part of the description, pages:

1-15, filed with the letter of 05.06.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-14
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-14
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

ITEM I:

The sequence listing pages 1-15, filed with the letter of 05.06.2000 do not form part of the application (Rule 13ter.1(f) PCT).

ITEM II:

The priority appears to be validly claimed for the whole claimed subject-matter and the P-documents mentioned in the International Search Report therefore do not appear to be relevant. The applicant explains that the designation "fabG" used in the priority document and the designation "mabA" used in the application are equivalents and that the sole cause for making the amendment was the wish to use updated language.

ITEM V:

THE CLOSEST PRIOR ART:

5.1 The closest prior art is disclosed in "Abstracts of the Interscience Conference on Antimicrobial Agents and Chemotherapy, vol. 34, page 163, (1994)" (D1). D1 discloses a method for identification of mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis by application of automated DNA sequence analysis. In the method a smear test is first carried out followed by an identification of a species specific hsp65 polymorphism. Subsequently, mutations were found in specific regions of seven genes. The genes investigated in D1 are rpoB, katG, inhA, orf1, rpsL, gyrA and 16SrRNA.

NOVELTY:

5.2 The method of claim 1 differs from the method of D1 in that the rpoB primers are selected so that an amplification of a sequence from the rpoB gene is indicative of the presence of Mycobacterium tuberculosis and in that the 23S gene is among the genes sequenced at the same time as the species-specific sequence and in that the sequences of the PR, embB, pncA and gyrA sequences are determined if the species specific test is positive. Thus, claims 1-13 are considered novel.

5.3 The only primer sequence of the present application, which is known from the prior art, is SEQ ID NO.1 which is disclosed in claim 22 of WO 95/33851 (D2). However, none of the combination of amplification and sequencing pairs are disclosed in the cited prior art documents. Claim 14 is therefore considered novel.

INVENTIVE STEP:

5.4 All of the genes mentioned in claims 1-3 of the present application are well-known as being involved in resistance development in Mycobacterium tuberculosis.

To the skilled person, who wishes to detect and characterize the resistance profile of a Mycobacterium tuberculosis sample isolated from an infected patient, it would therefore, prima facie, be obvious to test whether one or more of these genes carries a mutation. D1 informs the skilled person that mutations may be identified by sequencing of a number of genes. WO 97/23650 (D3) provides detailed information on how to sequence for polymorphisms.

WO 95/33074 (D2) discloses that PCR of a region of the rpoB gene may be used for both M.tuberculosis detection and drug susceptibility testing. The feature of claim 1 that the generation of rpoB sequences is indicative of the presence of M.tuberculosis therefore does not contribute to the inventiveness of claim 1. However, none of the cited documents suggest that the sequence of the 23S gene is useful in species determination of other Mycobacteria strains, in case the test for M.tuberculosis is negative. The method of claim 1 therefore solves the problem of providing in a single sequencing procedure the possibility of detecting and drug susceptibility testing M.tuberculosis and alternatively identifying another mycobacterium strains. In the absence of any suggestion in the prior art to include the 23S gene in the genes to be analysed firstly in a hierachical method, claim 1 is considered inventive.

Claims 2-13 which depend on claim 1 are considered inventive as well.

5.5 The use of primers for amplification of genes involved in antibiotic resistance is disclosed in D1 and is not considered inventive. The special combinations of amplification primers and sequencing primers (a) - (j) mentioned in claim 14 are not suggested in the prior art, but the sets of primers, per se, do not appear to

overcome a problem of the prior art documents which disclose methods for determining the sequence of several M.tuberculosis genes. The applicant argues that some primers perform better than others and considers the primers mentioned in claim 14 to perform very well. However, nothing in the present application suggests that any of the sets of primers solve a technical problem in an unexpected way. Nevertheless, the inclusion of 23S in the genes to be sequenced is not suggested in D1 and given the advantage obtained by its inclusion, present claim 14 is considered inventive.

INDUSTRIAL APPLICABILITY:

5.6 Present claims 1-14 are considered industrially applicable.

ITEM VII:

7.1 Contrary to the requirements of Rule 5(a)(ii) PCT, the discussion of the closest prior art document D1 does not reflect all the relevant background art disclosed therein.

7.2 It is not possible to incorporate the teaching of a prior art document into the present application's disclosure by the expression "herein incorporated by reference" or equivalents thereof (see p.4) (cf PCT Guidelines, C-II, 4.17).

ITEM VIII:

8.1 It is not clear from claim 3 under which conditions the third sequencing procedure is carried out, if the mere presence of M.tuberculosis is sufficient to give rise to the third sequencing procedure, then the prerequisites for carrying out the third and second sequencing procedures are the same and the hierarchical principle of the invention as explained in the application is not observed.

8.2 The object of the invention would only appear to be met if the kit contains primers which amplify the rpoB gene in a way so that the amplification of the rpoB gene is indicative of the presence of M.tuberculosis. At present this is not a requirement of claim 14.

8.3 It is not clear from the application whether the sequencing of a special region of

the 23S gene is needed in order to identify other strains than M.tuberculosis.